THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 266, No. 7, Issue of March 5, pp. 4067–4073, 1991
Printed in U.S.A.

Demonstration of the Phosphorylation of Dihydropyridine-sensitive Calcium Channels in Chick Skeletal Muscle and the Resultant Activation of the Channels after Reconstitution*

(Received for publication, September 18, 1990)

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We have examined the effects of cAMP elevating agents on the phosphorylation of dihydropyridine-sensitive Ca^{2+} channels in intact newborn chick skeletal muscle. *In situ* treatment with the β-adrenergic receptor agonist isoproterenol resulted in the phosphorylation of the 170-kDa α_1 subunit in the intact cells, as evidenced by a marked decrease in the ability of the α_1 peptide to serve as a substrate in *in vitro* back phosphorylation reactions with [γ-^{32}P]ATP and the purified catalytic subunit of cAMP-dependent protein kinase. The phosphorylation of the 52-kDa β subunit was not affected. The effects of isoproterenol were time- and concentration-dependent and were mimicked by other cAMP elevating agents but not by the Ca^{2+} ionophore A23187 or a protein kinase C activator. To test for functional effects of the observed phosphorylation, purified channels were reconstituted into liposomes containing entrapped fluo-3, and depolarization-sensitive and dihydropyridine-sensitive Ca^{2+} influx was measured. Channels from isoproterenol-treated muscle exhibited an increased rate and extent of Ca^{2+} influx compared to control preparations. The effects of isoproterenol pretreatment could be mimicked by phosphorylating the channels with cAMP-dependent protein kinase *in vitro*. These results demonstrate that the α_1 subunit of the dihydropyridine-sensitive Ca^{2+}-channels is the primary target of cAMP-dependent phosphorylation in *intact* muscle and that the phosphorylation of this protein leads to activation of channel activity.

Voltage-dependent Ca^{2+} channels of the L type, *i.e.* those that exhibit slow, long-lasting currents and high affinity for dihydropyridines, are regulated in cardiac and skeletal muscle, as well as in other cells, by cyclic AMP-dependent events (1–7). Electrophysiological studies suggest that this regulation occurs via a pathway that involves phosphorylation of the channels or associated regulatory proteins (4, 5). However, no evidence has been obtained from biochemical studies using intact cells to elucidate what phosphorylation reactions occur. Methods have been developed recently to allow for the isolation of L-type Ca^{2+} channels from skeletal (8–15) and cardiac muscle (16). Evidence suggests that the channels may be multisubunit proteins, but in each case peptides referred to as the α_1 subunits are the major functional components of the channels. These α_1 peptides have structures related to other voltage-dependent channels (17, 18), and contain potential phosphorylation sites (12, 14, 21–27), as well as the binding domains for the multiple chemical classes of drugs that can inhibit or activate the channels (10–16, 19). Expression of these α_1 subunits in the absence of other putative subunits results in the expression of functional Ca^{2+} channels in *Xenopus* oocytes or mammalian cells (18, 20). The roles of other putative subunits remain unknown.

Studies have been performed *in vitro* with the purified skeletal muscle Ca^{2+} channels and purified protein kinases in order to attempt to elucidate the potential sites for regulation of the channels by receptor dependent events. The results of these studies demonstrated that the 170-kDa α_1 peptide and a 52-kDa β peptide are substrates *in vitro* for cAMP-dependent protein kinase (12, 14, 21–26), protein kinase C (24, 27), Ca/calmodulin-dependent protein kinase II (25), casein kinase II (26), and an unidentified protein kinase present in skeletal muscle membranes (23). However, whether or not any or all of these reactions occur in intact cells has not been determined. In the present study we have assessed the effects of cAMP-elevating agents on the phosphorylation of the putative subunits of Ca^{2+} channels in intack chick skeletal muscle and determined the functional effects of this phosphorylation by using reconstitution methodology.

MATERIALS AND METHODS

Materials—Newborn chicks were purchased from Northern Hatcheries (Beaver Dam, WI) or hatched in house. [γ-^{32}P]ATP and the dihydropyridine (+)[^{3}H]PN 200-110 were purchased from Amersham Corp. Digitonin (AnalaR grade) was purchased from Gallard-Schlesinger (Carle Place, NY). The catalytic subunit of cAMP-dependent protein kinase was purified to homogeneity from bovine heart (28). Protein kinase C was purified to homogeneity from chicken brain (30); the purified enzyme contained at least two isoforms (30). Isoproterenol, forskolin, A23187, isobutylmethylxanthine, 8-bromo-cAMP, L-α-phosphatidylcholine, cholesterol, dicetyl phosphate, valinomycin, protease inhibitors, and molecular weight markers were purchased from Sigma. Dioctanoyl glycerol was from Avanti (Pelham, AL), Extracti-Gel was from Pierce Chemical Co., and fluo-3 (free acid) was from Molecular Probes (Eugene, OR). All other materials were from commercial sources as previously described (16).

In Situ Phosphorylation Studies—Breast muscles (~1 cm long, 0.3 cm wide, 0.1 cm thick) from 1–3-day-old newborn chicks were dissected and placed into oxygenated Kreb's solution and equilibrated at 37 °C for approximately 30 min. The tissues were divided into groups (10 g each) and subjected to the experimental treatments described. Treatments were terminated by freeze-clamping the tissues with stainless steel Wollenberger tongs precooled to the temperature

* This work was supported by National Institutes of Health Grant HL-23306, a junior fellowship from the American Heart Association of Metropolitan Chicago (to C. F. C.), a postdoctoral fellowship from the Muscular Dystrophy Association of America (to C. M. W.), and a FPI fellowship from the Ministerio de Educacion y Ciencia of Spain (to L. M. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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of liquid nitrogen. The frozen wafers of tissue were pulverized under liquid nitrogen with a mortar and pestle and then added to 4 volumes of homogenization buffer (25 mM $NaKPO_4$, 50 mM NaF, 10 mM EDTA, 2 mM EGTA,[1] 0.3 M sucrose, and a protease inhibitor cocktail consisting of 1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, and 2 μg/ml leupeptin). The resulting frozen slurry was homogenized in an ice bath with a Polytron (PT-10 probe) at setting 7 for 5 × 20 with 30 s between each burst. Microsomes were prepared according to a modification of the method of Fernandez *et al.* (29). The yield of membranes was 1 mg of protein/g of tissue, and the content of Ca^{2+} channels was 5–10 pmol/mg protein based on the binding of the dihydropyridine (+)[^{3}H] PN 200-110.

Purification of Ca^{2+} Channels from Newborn Chick Skeletal Muscle—All solutions contained the protease inhibitor cocktail described above. The channel proteins were solubilized in digitonin and partially purified via a two-step procedure that utilized DEAE-Sephadex A-25 and wheat germ agglutinin-Sepharose affinity chromatography as described (16), except that the buffers were modified to include 25 mM $NaKPO_4$, pH 7.4, and 20 mM NaF to prevent dephosphorylation.

Back Phosphorylation Reactions with the Isolated Ca^{2+} Channels—Back phosphorylation was carried out using conditions similar to those used in previous studies of *in vitro* phosphorylation of isolated channels (25, 27). Partially purified channel proteins (40–60 μg protein) were incubated in the presence of 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 2 mM EGTA, 50 μM [γ-^{32}P]ATP (600–2500 cpm/pmol), and 0.25 μM catalytic subunit of cAMP-dependent protein kinase at 4 °C for 30 min. The reactions were stopped by adding two volumes of "stop buffer" (50 mM $NaKPO_4$, 50 mM NaF, and 20 mM EDTA) and protease inhibitors in 0.1% digitonin. Samples were then subjected to a two-step electrophoresis procedure (36) which provided a convenient additional purification step that facilitated detection of the channel proteins. First, samples were electrophoresed onto a nondenaturing polyacrylamide gradient gel (4–10% polyacrylamide) that contained 0.1% digitonin (36). Using this procedure, the channel protein migrated as a high molecular weight protein that could be identified as the channel by a variety of procedures including antibody staining and photoaffinity labeling. After a brief staining of the nondenaturing gel to locate the channel protein, the appropriate bands were excised, washed, and then loaded onto an SDS gel (31) containing a linear gradient of 5–15% polyacrylamide. Channel peptides were identified by Coomassie blue or silver staining.

The calculation of the stoichiometries of phosphorylation of the control samples were based on the specific activity of the [γ-^{32}P] ATP, the ^{32}P content of the excised band from the SDS gel, and the concentration of dihydropyridine receptors applied to the gel as measured by the binding of (+)[^{3}H]PN 200-110 as previously described (25, 27). The calculation of the stoichiometries of the isoproterenol-induced phosphorylations were based on the percentage inhibition of the *in vitro* phosphorylation that occurred as a result of the phosphorylation that occurred *in situ*.

Preparation of Liposomes and Reconstitution of Purified Channels—A mixture of L-α-phosphatidylcholine/cholesterol/dicetyl phosphate (7:3:0.005) was sonicated in "internal medium" (145 mM KCl, 50 mM KF, 0.5 mM EGTA, 20 mM Hepes, pH 7.2) in a Branson 1200 bath sonicator for 30 min at 4 °C. Extracti-Gel columns were equilibrated with internal medium containing the sonicated lipids (1.2 mg/ml) 2 μM Bay K8644 and 2 mM $MgCl_2$. The reconstitution procedure consisted of two steps. In the first step the purified Ca^{2+} channels were reconstituted into small liposomes, and in the second step these liposomes were fused with larger liposomes containing the Ca^{2+} indicator dye fluo-3. For step one, the partially purified Ca^{2+} channels were mixed with the sonicated lipids and passed through Extracti-Gel columns to remove the detergent. For step two, larger proteoliposomes suitable for flux studies were prepared by suspending a dried film of the lipids (12 mg) indicated above in 1 ml of buffer containing internal medium, 20 μM fluo-3 free acid, 2 μM Bay K8644, 2 mM $MgCl_2$ and 25 μg of the reconstituted channels obtained from step one. Several small glass beads were added to the mixture and a suspension was prepared by vigorous agitation using a Vortex mixer for 2 min (38). The liposomes were kept 1 h on ice and then passed through a Sephadex G-50 column (20 × 1 cm) equilibrated with "external medium" (145 mM NaCl, 50 mM NaF, 0.5 mM EGTA, 20 mM Hepes, pH 7.4), Bay K8644 (2 μM), and $MgCl_2$ (2 mM). This step diluted the proteoliposomes to 3 ml. The preparations were kept on ice until flux measurements were made. The efficiency of the reconstitution was determined in parallel experiments in which channels were first prelabeled with [^{3}H]PN 200-110; by this criteria, we found that ~60% of the channels were incorporated into the fluo-3-containing liposomes.

In some experiments the purified channels were previously phosphorylated with cAMP-dependent protein kinase before the first reconstitution step with the Extracti-Gel procedure. For these experiments, the channels were phosphorylated as described in the section on back phosphorylation except that nonradioactive ATP was used.

Calcium Influx Measurements—Ca^{2+} influx was measured using fluo-3 as the Ca^{2+} indicator dye. Measurements were made in stirred cuvettes at room temperature with a fluorescent spectrophotometer (Perkin-Elmer, model LS-5B); excitation and emission wavelengths were 490 and 530 nm, respectively, and slits were 10 nm. Aliquots of 0.1 ml of proteoliposomes were added to 1.9 ml of "external buffer" and Ca^{2+} influx was induced by addition of 5 mM $CaCl_2$ to the cuvette. Membrane potential was achieved by establishing a K^+ gradient across the vesicle membrane through the use of valinomycin (0.1 μM) (39). In the absence of a K^+ gradient, no valinomycin sensitive Ca^{2+} influx was observed. Liposomes devoid of protein were used as controls to define the amount of flux due to the liposomes alone. None of the Ca^{2+} channel activators or inhibitors tested exhibited any effects on Ca^{2+} influx in protein free liposomes or in liposomes containing channels that were purified and reconstituted in the absence of Bay K8644. Calibration of the fluo-3 signal was performed using ionomycin (10 μM) in the presence of Mn^{2+} or Zn^{2+} (2 mM each) to define the signals corresponding to 100 and 500 nM Ca^{2+}, respectively (40).

RESULTS

Effect of Isoproterenol on Phosphorylation of Ca^{2+} Channel Components in Newborn Chick Skeletal Muscle—To study the phosphorylation of Ca^{2+} channels in intact muscle, we used the technique of back phosphorylation in which phosphorylation is allowed to occur in the intact muscle with the unlabeled endogenous ATP pools, and then the proteins are isolated and subjected to *in vitro* phosphorylation with labeled ATP and purified cAMP-dependent protein kinase. Under these conditions, if phosphorylation occurred in the intact cells at the same site as that phosphorylated by the protein kinase used in the subsequent *in vitro* phosphorylation reactions, then the protein should be a poor substrate in the later reaction. This technique of "back phosphorylation" has been successfully used to study the *in situ* phosphorylation of other proteins, including the structurally related voltage-dependent Na^+ channels (32).

To determine the effect of isoproterenol on channel phosphorylation *in situ*, muscle was incubated with either no drug or with 0.1 mM isoproterenol for 30 min. Membranes were prepared and the Ca^{2+} channels were isolated under conditions to prevent dephosphorylation. The isolated channels were subjected to the back phosphorylation reaction with the catalytic subunit of cAMP-dependent protein kinase, and the phosphorylated proteins were analyzed by SDS-gel electrophoresis. As can be seen in Fig. 1*A*, equal amounts of protein were resolved on the SDS-gels containing the samples from the control and isoproterenol (ISO)-treated muscle. The corresponding autoradiogram depicting the phosphoproteins (Fig. 1*B*) shows that the isoproterenol treatment led to a marked decrease in the ability of the α_1 subunit of the channels to serve as a substrate in the back-phosphorylation reaction (Fig. 1*B*). In contrast to the results obtained for the α_1 subunit, no decrease due to the isoproterenol treatment was observed in the phosphorylation of the β subunit (Fig. 1*B*). These results suggested that the isoproterenol treatment led to a selective phosphorylation of the α_1 subunit, but not the β subunit, in the intact muscle. These experiments were performed at least 20 times, and ISO effects similar to those

[1] The abbreviations used are: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; ISO, isoproterenol; diC8, dioctanoyl glycerol.

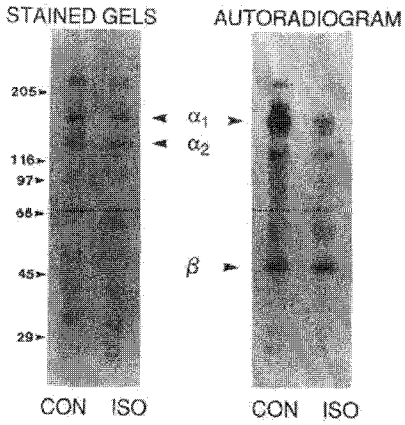

FIG. 1. **Effect of isoproterenol on the phosphorylation of Ca^{2+} channels in intact chick skeletal muscle.** Newborn chick skeletal muscle was incubated in oxygenated Kreb's solution in the absence (*CON*) or in the presence of 100 μM isoproterenol (*ISO*) for 30 min. The tissues were freeze-clamped and microsomes were isolated as described under "Materials and Methods." Ca^{2+} channels were then partially purified and back-phosphorylated with cAMP-dependent protein kinase and [γ-^{32}P]ATP and then subjected to successive nondenaturing and SDS-gel electrophoresis. *A*, Coomassie blue-stained SDS gels demonstrating that equal amounts of protein from each treatment were loaded onto the gels. *B*, autoradiogram of gel shown in *A*. Note that the *in situ* treatment with isoproterenol resulted in a marked decrease in the ability of the 170-kDa α_1 peptide to serve as substrate in the *in vitro* phosphorylation reaction with cAMP-dependent protein kinase, whereas no change was observed for the 52-kDa β peptide. Similar results were obtained in at least 20 experiments.

shown were obtained in each experiment.

In addition to the phosphorylated 170-kDa α_1 and 52-kDa β subunits observed in the autoradiogram shown in Fig. 1*B*, two other phosphoproteins were observed. One corresponded to a stained band that had a mobility of approximately 220 kDa (Fig. 1*A*). The phosphorylation of this protein was also stimulated by the *in situ* isoproterenol treatment (Fig. 1*B*). Another phosphoprotein had a mobility of ~130 kDa, and migrated slightly faster than the α_2 peptide (Fig. 1*B*), but did not correspond to a major stained protein (Fig. 1*A*). That this phosphopeptide was not the 140-kDa α_2 subunit of the Ca^{2+} channel was readily demonstrated by using sucrose density gradient centrifugation to separate this phosphoprotein from the α_1 and α_2 peptides. Using this procedure, the α_1 and α_2 peptides were easily separated from the 130-kDa phosphoprotein (data not shown).

Time Course of Isoproterenol-induced Phosphorylation—The time course of the isoproterenol-induced phosphorylation of the channels *in situ* was determined by treating muscle with the drug for various periods of time and then isolating and analyzing the channels as described above. The results depicting the phosphorylation of the α_1 subunit are presented graphically in Fig. 2. Half-maximal phosphorylation occurred between 1 and 2 min and reached maximal levels by 5 min (Fig. 2). The phosphorylation could be sustained during a 30-min treatment with the agonist. No significant effects of ISO on the *in situ* phosphorylation of the β peptide was observed at any time period tested. We estimated that the isoproterenol treatment resulted in a stoichiometry of ~1.4 mol of phosphate incorporation per mol of α_1 peptide. This is based on the observation that the α_1 peptide isolated from control tissue incorporated 2.0 mol of phosphate per mol of protein in the *in vitro* reaction, similar to previous reports (25, 27); the isoproterenol treatment decreased this by 69% (Fig. 2). These

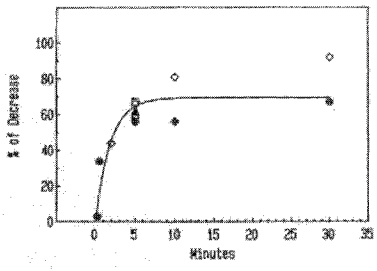

FIG. 2. **Time course of stimulation of Ca^{2+} channel phosphorylation by isoproterenol.** Experiments were performed as described in the legend to Fig. 1 except that the time of the isoproterenol treatment was varied as shown. The bands corresponding to the α_1 peptide were excised and the ^{32}P content was determined by scintillation counting. The results are expressed as a percentage decrease in the *in vitro* back-phosphorylation with respect to control. The results shown are from several experiments performed independently; the *different symbols* represent data obtained from independent experiments.

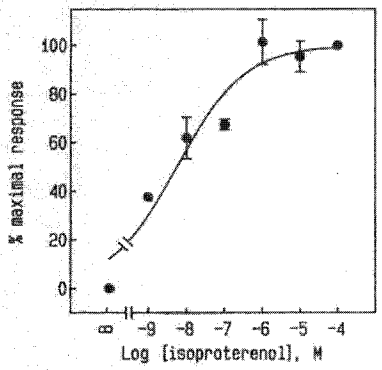

FIG. 3. **Dose-response relationship for the isoproterenol-induced effects on Ca^{2+} channel phosphorylation in newborn chick skeletal muscle.** Experiments were performed as described in the legend to Fig. 1 except that the time of exposure was 5 min and the doses of isoproterenol were varied as indicated. The results shown are means ± standard errors obtained from three to five separate experiments, except the value for 10^{-9} which was from a single determination.

conclusions are based on the results from several independent experiments shown in Fig. 2.

Dose-response for Isoproterenol—To demonstrate that the effects of isoproterenol could be elicited in a dose-dependent manner, we exposed muscle to varying concentrations of the agonist for 5 min. The channels were isolated and their state of phosphorylation analyzed (Fig. 3). Dose-dependent effects of ISO were observed; the EC_{50} was calculated to be ~10 nM, and maximal effects were obtained at 1 μM. These values are consistent with other effects of isoproterenol that are mediated through activation of β-adrenergic receptors.

Effects of Cyclic AMP Elevating Agents—The effect of isoproterenol on the phosphorylation of the Ca^{2+} channels was likely to be mediated by elevations in intracellular cAMP and activation of cAMP-dependent protein kinase. To obtain further evidence that increases in cAMP led to the observed effects on the phosphorylation of the channels, we tested other cAMP elevating agents for their ability to induce phosphorylation of the α_1 peptide *in situ*. The adenylyl cyclase activator forskolin, the phosphodiesterase inhibitor isobutylmethylxanthine, and 8-bromo-cAMP produced effects qualitatively and quantitatively similar to isoproterenol (Fig. 4). These results strongly suggested that cAMP-dependent protein kinase phosphorylated the channels *in situ* and thus caused a decrease in their ability to serve as substrates for

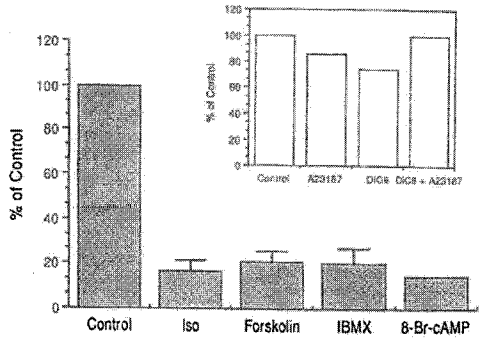

FIG. 4. **Effects of different agents that elevate cAMP on the phosphorylation of the α_1 subunit *in situ*.** *Main graph,* chick skeletal muscle was incubated for 30 min without (*Control*) or with isoproterenol (*Iso*, 10 μM), forskolin (10 μM), isobutylmethylxanthine (*IBMX*, 10 μM), or 8-Br-cAMP (1 mM). Ca^{2+} channels were isolated as described and phosphorylation of the α_1 peptide after back-phosphorylation was quantified by scintillation counting of bands visualized by autoradiography. The results are expressed as a percentage inhibition of ^{32}P incorporation in the back-phosphorylation reaction relative to control. The results shown are the mean ± S.D. for two experiments, except that the data for the 8-Br-cAMP were from a single determination. *Inset,* similar experiments were performed except that the treatments were with A23187 (10 μM), diC8 (100 μM), or A23187 plus diC8 as indicated. The results shown are from one experiment that was repeated twice with similar results.

this kinase in the *in vitro* reactions.

However, it is possible that another kinase might participate in the *in situ* reactions. Conceivably, ISO or the cAMP elevating agents might cause an increase in intracellular Ca^{2+} that might activate a Ca^{2+}-dependent kinase. Previous studies have established that protein kinase C and a Ca^{2+}/calmodulin-dependent protein kinase can phosphorylate the α_1 subunit *in vitro* both at sites distinct from and shared with those phosphorylated by cAMP-dependent protein kinase (25, 27). Therefore, to test if activation of a Ca^{2+}-dependent kinase or protein kinase C *in the intact muscle* could cause the α_1 subunit to be a poorer substrate for cAMP-dependent protein kinase in the back-phosphorylation reaction, we exposed muscle to the Ca^{2+} ionophore A23187 (10 μM) and/or to dioctanoyl glycerol (diC8, 100 μM), a synthetic diacylglycerol that activates protein kinase C. Neither agent, alone or in combination, significantly affected the ability of the α_1 subunit to serve as a substrate in the back-phosphorylation reaction with cAMP-dependent protein kinase. The phosphorylation of the samples from the A23187- and/or diC8-treated muscle was the same as the controls (Fig. 4, *inset*). Although these agents did not affect the back-phosphorylation of the α_1 subunit by cAMP-dependent protein kinase, the concentration of diC8 used in these experiments should have activated protein kinase C as 0.1 mM diC8 was previously shown to mimic the effects of phorbol esters on cardiac functions and to exert maximal effects on heart rate and force of contraction in perfused rat hearts (41). Similarly, the concentration of A23187 that we used was higher than concentrations routinely used to demonstrate Ca^{2+}-dependent effects in many systems (42, 43). In another experiment we tested the effects of phorbol myristate acetate and found that it also produced no effects in the back-phosphorylation assay.

As a further test of the cAMP-dependence of the effect of isoproterenol, we subjected channels from control and ISO-treated muscle to back-phosphorylation with purified protein kinase C. Phosphorylation with protein kinase C was performed with the isolated membranes as described (27) and the channels purified and analyzed. Protein kinase C incorporated ~2 mol of phosphate per mol of protein into the α_1 subunit (data not shown), in agreement with previous studies (27). No difference was observed between the samples from the control and ISO-treated preparations (data not shown) indicating that protein kinase C did not phosphorylate the channels in response to the ISO treatment. Taken together, these results provided further evidence that the effects of ISO and the cAMP elevating agents were due to phosphorylation of the Ca^{2+} channels *in situ* by cAMP-dependent protein kinase.

Functional Consequences of the Isoproterenol Treatment on Ca^{2+} Channel Activity—In order to demonstrate that the isoproterenol-induced phosphorylation of the α_1 subunit had functional consequences on channel activity, we used a reconstitution method to study Ca^{2+} influx through the partially purified Ca^{2+} channels. Several previous studies have reported reconstitution of Ca^{2+} channels from detergent solubilized membranes (44, 45) or purified preparations (46–48) and the subsequent measurement of Ca^{2+} influx using either $^{45}Ca^{2+}$- or Ca^{2+}-sensitive dyes. In two of these studies the effects of *in vitro* phosphorylation with protein kinase A were reported (44, 48).

The method we used was similar in concept and involved reconstituting the partially purified channels into liposomes and fusing these liposomes with larger liposomes containing the Ca^{2+} indicator dye fluo-3 (40). Using this approach we were able to demonstrate the reconstitution of channels that were sensitive to depolarization and dihydropyridines. Liposomes containing no protein were used as controls, and the passive entry of Ca^{2+} into these liposomes was not sensitive to either valinomycin-induced depolarization (Fig. 5, *bottom trace*) or the dihydropyridines. In preliminary experiments we found that channels which were purified and reconstituted in the absence of the Ca^{2+} channel activator Bay K8644 exhibited little activity upon reconstitution, in agreement with an earlier report (46). However, liposomes containing Ca^{2+} channels which were purified and reconstituted in the presence of

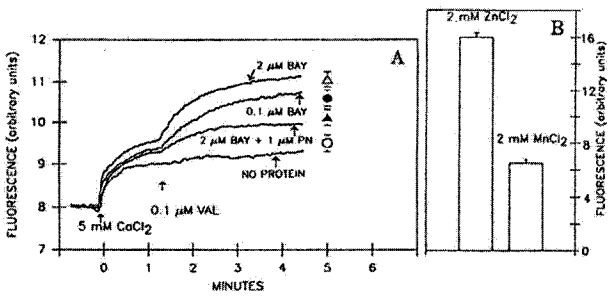

FIG. 5. **Activity of partially purified Ca^{2+} channels after reconstitution into liposomes containing the Ca^{2+} indicator dye fluo-3.** Liposomes containing no protein (controls) or partially purified channels were prepared as described under "Materials and Methods." Ca^{2+} influx was initiated with the addition of 5 mM Ca^{2+} at time 0, and depolarization was achieved by the addition of valinomycin at the time indicated. The results shown are from a representative experiment, and the *symbols* at the end of the *traces* show the mean ± S.E. for the maximum fluorescence obtained in three similar experiments. The actual mean changes in fluorescence (in arbitrary units) *over baseline* were: no protein, 1.5 ± 0.2 (○); channels plus 2 μM Bay K8644, 3.0 ± 0.2 (△); channels plus 0.1 μM Bay K8644, 2.5 ± 0.2 (●); and channels plus 2 μM Bay K8644 and 1 μM PN 200-110, 2.2 ± 0.1 (▲) ($n = 3$). In *B* are shown fluorescence values obtained after calibration with 10 μM ionomycin and either 2 mM $MnCl_2$ or $ZnCl_2$. The fluorescence obtained in the presence of Mn^{2+} was corrected for the autofluorescence of the liposomes (0 in this graph) and was used to obtain F_{max} and F_{min} (40). The validation of this method was assessed when the predicted level for 500 μM Ca^{2+} was coincident with that obtained with 2 mM Zn^{2+} (40).

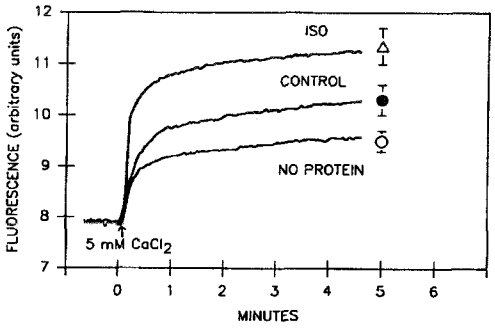

FIG. 6. **Effect of *in situ* treatment of intact muscle with isoproterenol on the activity of the partially purified Ca^{2+} channels after reconstitution.** Muscle was treated with isoproterenol (*ISO*) or not (*Control*) and Ca^{2+} channels were isolated and reconstituted as described under "Materials and Methods." Bay K8644 (0.1 μM) and valinomycin were present in all samples for the entire assay period. The results shown are from a representative experiment that was repeated four times with similar results. The mean increases in fluorescence over baseline were: no protein, 1.5 ± 0.2 (○); control channels, 2.3 ± 0.3 (●); channels exposed to isoproterenol *in situ* (*ISO*), 3.3 ± 0.3 (△) ($n = 4$).

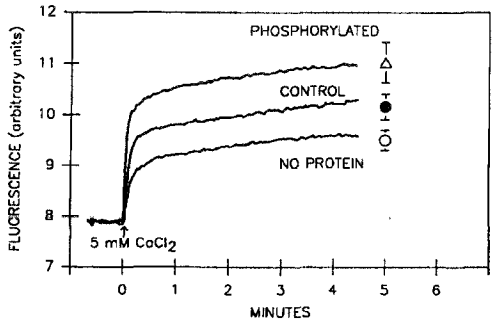

FIG. 7. **Effect of *in vitro* phosphorylation with cAMP-dependent protein kinase on Ca^{2+} channel activity after reconstitution.** Partially purified Ca^{2+} channels were phosphorylated with 0.1 μM of the catalytic subunit of cAMP-dependent protein kinase and 50 μM ATP as described under "Materials and Methods." The channels were then reconstituted and channel activity was measured as described in the legends to Figs. 5 and 6. The results shown are from a typical experiment from a group of 3. The mean increase in fluorescence over baseline were: no protein, 1.5 ± 0.2 (○); control channels, 2.2 ± 0.2 (●); phosphorylated channels, 2.9 ± 0.3 (△) ($n = 3$).

Bay K8644 showed Ca^{2+} uptake that was somewhat greater than the no protein liposomes. Ca^{2+} influx in these liposomes was increased 2-fold upon the addition of valinomycin (Fig. 5, *second trace from top*). The valinomycin-sensitive Ca^{2+} influx was totally dependent on the K^+ gradient (data not shown). Ca^{2+} influx was similar in the presence of 0 or 2.5 mM external K^+, but was markedly decreased when the external K^+ concentration was increased further, and was abolished at 145 mM K^+.[2]

The valinomycin-sensitive influx was further increased by increasing the Bay K8644 to 2 μM (*top trace*) and this influx was inhibited by 60% by 1 μM (+)PN 200-110, a dihydropyridine Ca^{2+} channel blocker (Fig. 5, *third trace from top*). (It should be noted that, unless otherwise indicated, 0.1 μM Bay K8644 was present in all reconstitutions, because 2 μM Bay K8644 was added to the reconstitution buffers and became decreased to 0.1 μM upon dilution of the samples into the cuvettes.) The calibration of the Ca^{2+} signal was performed in the presence of ionomycin and Mn^{2+} or Zn^{2+} as suggested by Kao *et al.* (40) and indicated that the maximal increments in fluorescence observed (valinomycin plus 2 μM Bay) with the channel containing liposomes amounted to a rise in intervesicular Ca^{2+} of ~145–155 nM over values of 60–70 nM in liposomes alone. These results established that the reconstitution method used allowed for the detection of Ca^{2+} influx with the characteristics expected for a depolarization-sensitive, dihydropyridine-sensitive Ca^{2+} channel.

We next determined if the channels isolated from the isoproterenol-treated muscle exhibited properties in the reconstitution assays different from those isolated from control muscle. All experiments were performed by isolating channels under conditions to prevent dephosphorylation and the fluo-3 studies were performed in the presence of 50 mM NaF (external) or KF (internal) for the same purpose. The results of these studies indicate that the *in situ* treatment with ISO led to an increased rate and extent of Ca^{2+} influx in the reconstitution assay performed with channels that had been exposed to valinomycin 1 min before the addition of Ca^{2+}. A representative experiment is shown in Fig. 6 and the *symbols* at the end of the *traces* show the means ± standard errors for the maximum fluorescence observed in four experiments. The channels isolated from the ISO-treated muscle exhibited a 2.3-fold increase in maximum Ca^{2+} influx over the controls.

If the effects of the *in situ* treatment with isoproterenol were due to phosphorylation by cAMP-dependent protein kinase, then one would expect that the effects of the ISO treatment could be mimicked by phosphorylating the channels *in vitro* with cAMP-dependent protein kinase. Therefore, we isolated the Ca^{2+} channels from control microsomal membranes, phosphorylated the purified channels *in vitro* with the catalytic subunit of cAMP-dependent protein kinase, and then reconstituted the channels for activity determinations. The results of a representative experiment are shown in Fig. 7 and demonstrate that the *in vitro* phosphorylation qualitatively and quantitatively mimicked the effects of the *in situ* treatment with isoproterenol. The increase in fluorescence obtained 5 min after the addition of Ca^{2+} with samples from three similar experiments is shown by the *symbols* at the end of the *traces* (Fig. 7). The mean increase in fluorescence due to the *in vitro* phosphorylation was 2-fold.

DISCUSSION

The major findings of the present study are: (i) treatment of intact skeletal muscle with isoproterenol or other cAMP elevating agents led to the phosphorylation of the α_1 subunit of dihydropyridine-sensitive Ca^{2+} channels, and (ii) the observed *in situ* phosphorylation of the α_1 subunit led to Ca^{2+} channel activation. These results document that phosphorylation of dihydropyridine-sensitive Ca^{2+} channel components occurs in intact skeletal muscle. Taken together, the evidence supports the hypothesis that the α_1 subunit is the target of cAMP-dependent protein kinase *in situ* and that the regulation of channel activity by elevations in cAMP occurs at the level of this protein. This suggestion is strongly supported by our finding in the reconstitution studies that the same treatment that led to the phosphorylation of the α_1 subunit also resulted in an increased rate and extent of Ca^{2+} influx through the reconstituted channels.

The results supporting the view that the α_1 subunit is the target of cAMP-dependent phosphorylation in intact muscle cells complement findings from previous studies in which this peptide was found to be an excellent substrate for this kinase *in vitro* (21, 25, 26). In contrast, the present results which showed that no phosphorylation of the β peptide could be detected in the *in situ* studies in response to isoproterenol or

[2] L. M. Gutierrez and M. M. Hosey, manuscript in preparation.

the other cAMP-elevating agents was not readily predicted because this peptide can be phosphorylated with cAMP-dependent protein kinase *in vitro* (21, 24, 26). However, we have found that the stoichiometry of phosphorylation of the β peptide *in vitro* is 5-fold lower than the α_1 peptide when phosphorylation is studied with native channels in transverse tubule membranes (33). On the other hand, other investigators have used the purified (in contrast to membrane-bound) channels as substrates and reported that the α_1 and β subunit were equally effective as substrates for cAMP-dependent protein kinase *in vitro* (21, 35). The present results imply that the phosphorylation of the β peptide is not required for the regulation of the channels by cAMP-mediated events since the channels isolated from ISO-treated muscle exhibited increased activity under conditions in which only the α_1 peptide was phosphorylated. However, future studies are required to substantiate this suggestion.

The studies presented in this report relied on the technique of back-phosphorylation to study the effects of elevating cAMP on the phosphorylation of the Ca^{2+} channels *in situ*. While it is highly desirable to directly demonstrate the phosphorylation of proteins in intact cell studies by labeling intracellular ATP stores with ^{32}P, we have had little success in adapting this technique for the study of dihydropyridine-sensitive Ca^{2+} channels in skeletal muscle. Nevertheless, the results obtained with the indirect method of back-phosphorylation strongly suggest that the channels were phosphorylated by cAMP-dependent protein kinase in the intact cells. A role for Ca^{2+}-dependent kinases or protein kinase C was ruled out by the inability of the Ca^{2+} ionophore A23187 or the diacylglycerol diC8 to affect the back-phosphorylation carried out in the presence of cAMP-dependent protein kinase, and by the lack of effect of the ISO treatment on the ability of the channels to serve as substrates for protein kinase C. Since previous studies have established that there are unique sites that can be phosphorylated by the cAMP-dependent protein kinase *in vitro*, these sites are likely candidates for phosphorylation *in situ*. The finding that the ISO-mediated activation of the channels (as determined in the reconstitution assays) could be directly mimicked by *in vitro* phosphorylation with the purified cAMP-dependent protein kinase also provided further evidence for the role of this enzyme in the ISO-mediated effect. Taken together the results provide new information concerning the mechanism of regulation of Ca^{2+} channels by protein phosphorylation.

The measurement of a depolarization-sensitive Ca^{2+} influx with reconstituted Ca^{2+} channels or membrane-bound channels in vesicles has been previously observed (45–47, 49) but deserves a comment. In the reconstitution studies reported here, before the addition of valinomycin, the vesicles were depolarized (there was no membrane potential) but the channels were closed or only minimally active, despite the presence of Bay K8644. Similar observations were made by Dunn (49) who measured Ca^{2+} efflux through dihydropyridine-sensitive Ca^{2+} channels in transverse tubule membrane vesicles. Dunn reasoned (49) that the absence of channel opening under these depolarizing conditions was caused by the well known inactivation that occurs upon the chronic exposure of the channels to depolarizing conditions during the isolation of the channels. When the valinomycin was added, it established a K^+ gradient that first hyperpolarized the vesicles, but as the K^+ ran down its gradient the vesicles were subjected to a relative depolarization. The repolarization of the channels probably allowed them to pass from an inactivated state to a state where they opened in response to the depolarization. Under these conditions, the degree of Ca^{2+} influx that is observed is greatly augmented by the presence of Bay K8644. Similar phenomena have been observed by others (46, 47, 49) and the results of the present work suggest that the purified channels retain their properties of depolarization-sensitive activation and inactivation.

Acknowledgments—We gratefully acknowledge the assistance of the other members of our laboratory in the preparation of the skeletal muscle for the *in situ* assays.

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